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Flow cytometry

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An abbreviated version of this protocol was published in Science Immunology in Sep 2020

Type I interferon signaling in fibroblastic reticular cells prevents exhaustive activation of antiviral CD8+ T cells

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Detailed protocol

Flow cytometry of stromal cells and hematopoietic cells from lymph nodes

Reagents

- PBS
- RPMI-1640 medium (Sigma-Aldrich, cat. no. R8758)
- Fetal Calf Serum (Biowest, cat. no. S1810-050)
- Penicillin-streptomycin (Lonza, cat. no. DE17-602E)
- HEPES buffer solution (PAN Biotech, cat. no. P05-01500)
- EDTA-2Na (Sigma-Aldrich, cat. no. E5134)
- Collagenase P (Roche, cat. no. 12138730001)
- DNase I (Sigma-Aldrich, cat. no. 4527)
- Dispase (Roche)

Equipment

- Flow cytometer
- Constant-temperature incubator/shaker
- 15-ml tube (BD Falcon, cat. no. 352096)
- 50-ml tube (BD Falcon, cat. no. 352070)
- 1.5 ml tube (Biomedical sciences, cat. no. BC-MPF-150C)
- 5-ml polystyrene round-bottomed FACS tubes (BD Biosciences, cat. no. 352058)
- 1 ml cut tips
- 24 well culture plates (TPP, cat no. 009224)
- 96 well round bottom (Greiner Bio-one, cat. no. 7.650101)
- 3 ml syringes (BD Plastipak)
- 26 G needles (Terumo)
- 70 µm nylon cell strainer (BD Falcon, cat. no. 340336)

Prepare:

A. Digestion medium
   RPMI 2% of FCS
   20mM HEPES
B. Digestion medium + enzymes
   Dispase (40 µg/ml)
   Collagenase P (1 mg/ml)
   DNAse (25 µg/ml)
   24 well plate with 1ml of Digestion medium without enzymes/well. One well is needed per lymph node.

Preparation of lymph node stromal cells

1. Sacrifice the mice and harvest the lymph nodes. Transfer them in to the 24 well plate containing digestion medium without enzymes.
2. Replace the medium without enzymes with 1 ml of with pre-warmed digestion medium + enzymes (37°C).
3. Disrupt the lymph nodes using 26G needles to make small pieces.
4. Transfer the disrupted organs in to a fresh 15ml Falcon tube using cut tips.
5. Incubate 15 min in 37°C water bath; do not incubate cells for longer

5. Incubate 10 min in 37 °C water bath, do not incubate cells for longer!
6. During the incubation, prepare another set of fresh 15mL Falcon tubes with 5ml of MACS buffer (2% FCS, 3mL of EDTA in PBS 1X). Place the tubes on ice.
7. After incubation, disrupt gently the tissue by pipetting up and down using 1 ml cut tips (approx. seven times). Wait 2-3 minutes to let the nondigested tissue go to the bottom of the tube. Transfer the supernatant to the tube containing MACS buffer. Refill the tube with 1 ml of pre-warmed digestion medium + enzymes.
8. Incubate 10 min in the water bath and repeat step 7 until no pieces of tissue are visible. Normally it takes three rounds of digestion to get a single cell suspension. After the second round of digestion, normal 1 ml tips can be used for tissue dissociation.
9. Centrifuge the tubes 1200 RPM 5 min.
10. Discard the supernatant and resuspend the pellet in 1ml of MACS buffer.
11. Add 50 µL of anti-CD45 and anti-Ter119 MACS beads.
12. Incubate 20 min on ice.
13. Wash with 5 ml of MACS buffer and centrifuge at 1200 RPM 5 min.
14. In the meantime, activate the MACS columns with 3 ml of MACS buffer. Add collection tubes in the columns. Important the negative population contains the stromal cells.
15. Resuspend the cells in 5 ml of MACS buffer and add the cells to the top of the MACS column.
16. Wash 2 times with 3 ml of MACS buffer. Collect the negative population (Stroma cells)
17. Centrifuge the negatively selected cells (CD45 ter119 depleted cells) 1200 RPM 5 min. Discard the supernatant.
18. Stain the cells with the viability dye 1:1000 in PBS for 20 min at 4 °C. Wash the cells by adding 5 ml of MACS buffer. Centrifuge the samples at 1200 RPM for 5 min. discard the supernatant.
19. Incubate the cells for 20 minutes at 4 °C with the desired labelled antibodies diluted in MACS buffer. See Table S1
20. Wash the cells by adding 5 ml of MACS buffer and centrifuge the samples 1200 RPM 5 min.
21. Cells are ready for flow cytometric analysis.

Preparation of hematopoietic cells

1. Sacrifice the mice and harvest the lymph nodes. Transfer them in to the 15 ml Falcon tubes containing 10 ml of PBS.
2. Smash the organs through a 70 µm filters using a 3 ml syringe plunger.
3. Transfer the cells to a 15 ml Falcon tube.
4. Centrifuge the 10 ml cell suspension at 1500 RPM 5 min.
5. Cell are ready for staining. (Check Table S1 to adapt your staining of interest).

Flow cytometry analysis shown in the manuscript was performed a FACS Canto or a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Treestar Inc.)

Table S1. Antibodies used in the study.

Clone	Reagent	Conjugate	Dilution	Source
YN1/1.7.4	anti-ICAM-1	biotin	1:100	eBioscience
APB5	anti-CD140a	PE	1:100	eBioscience
6D5	anti-CD19	APC-Cy7	1:100	Biolegend
MEC13.3	anti-CD31	Alexa fluor-647	1:100	Biolegend
IA8	anti-Ly6G	PE	1:200	Pharmigen
AL-21	anti-Ly6C	PerCP	1:100	Biolegend
145-2C11	anti-CD3e	APC-Cy7	1:100	Biolegend
30F-11	anti-CD45	APC-Cy7	1:100	Biolegend
30F-11	anti-CD45	PerCP	1:100	Biolegend
RA3-6B2	anti-CD45R (B220)	APC-Cy7	1:100	Biolegend
53-6.7	anti-CD8a	APC	1:200	Biolegend
CI:A3-1	anti-F4/80	Alexa fluor-647	1:100	Biolegend
XMG1.2	anti-IFNγ	APC	1:100	Biolegend
8.1.1	anti-PDPN	PE	1:125	Biolegend
MP6-XT22	Anti-TNFα	FITC	1:100	Biolegend
TER-119	anti-Ter-119/Erytroid	APC-Cy7	1:100	Biolegend
IM7	anti-CD44	APC-Cy7	1:100	Biolegend
BP-3	anti-CD157	APC	1:100	Biolegend
M1/70	anti-CD11b	FITC	1:100	Biolegend
N418	anti-CD11c	APC-Alexa fluor-750	1:100	eBioscience
551	anti-SiglecH	APC	1:100	Biolegend
D7	anti-SCA1	PerCP	1:100	Biolegend
DAN11MAG	anti-Eomes	Alexa fluor-488	1:100	eBiosciences
4B10	anti-Tbet	PeCy7	1:100	Biolegend
MEL-14	anti-CD62L	BV-421	1:100	Biolegend

MIH5	PDL1	BV-711	1:100	BD horizon
RMP1-30	PD1	APC		Biolegend
429(MVCAM.A)	anti-CD106 (VCAM)	Alexa fluor-647	1:100	BD
RA3-6B2	anti-CD45R (B220)	Alexa fluor-647	1:200	Biolegend
	Streptavidin	BV-711	1:100	Biolegend
AF6-88.5.5.3	anti-H2Kb(MHC-I)	APC	1:200	Biolegend
M5/114.15.2	anti-I-A/I-E(MHC-II)	BV-421	1:100	Biolegend
2F1	anti-KLRG1	APC	1:1000	Biolegend

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1. Perez-Shibayama, C. , Gil Cruz, C. and Ludewig, B. (2020). Flow cytometry. Bio-protocol Preprint. bio-protocol.org/prep523.
2. Perez-Shibayama, C., Isler, U., Lütge, M., Cheng, H., Onder, L., Ring, S. S., Martin, A. D., Novkovic, M., Colston, J., Gil-Cruz, C. and Ludewig, B. (2020). Type I interferon signaling in fibroblastic reticular cells prevents exhaustive activation of antiviral CD8+ T cells. Science Immunology 5(51). DOI: 10.1126/sciimmunol.abb7066